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Crystallization and preliminary diffraction analysis of a β-galactosidase from *Trichoderma reesei*

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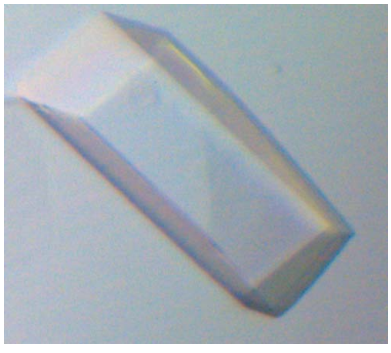

An extracellular β-galactosidase from *Trichoderma reesei* was crystallized from sodium cacodylate buffer using polyethylene glycol (PEG) as a precipant. Crystals grown by homogenous streak-seeding belonged to space group *P*1, with unit-cell parameters $a = 67.3$, $b = 69.1$, $c = 81.5$ Å, $\alpha = 109.1$, $\beta = 97.3$, $\gamma = 114.5^\circ$. The crystals diffracted to 1.8 Å resolution using a rotating-anode generator and to 1.2 Å resolution using a synchrotron source. On the basis of the Matthews coefficient ($V_M = 3.16$ Å^3 Da^{-1}), one molecule is estimated to be present in the asymmetric unit. The aim of the determination of the crystal structure is to increase the understanding of this industrially significant enzyme.

1. Introduction

β-Galactosidase (EC 3.2.1.23) is an enzyme that hydrolyses β(1–3)–β(1–4) galactosyl bonds in oligosaccharides and disaccharides. On the other hand, the enzyme also catalyses transglycosylation, the reverse reaction to the hydrolysis. The catalytic reactions of β-galactosidase occur inside an $(\alpha/\beta)_8$ barrel, in which two glutamic acid residues act as a proton donor and a nucleophile (Jenkins *et al.*, 1995). β-Galactosidase may also function as a lactase, which makes it an industrially important enzyme. It has many applications, *e.g.* the removal of lactose from dairy products (Kim *et al.*, 1997; Neelakantan *et al.*, 1999) and the production of lactulose, oligosaccharides and sulfated disaccharides (Murata *et al.*, 2001; Kim *et al.*, 2006; Sakai *et al.*, 2008). β-Galactosidase is often immobilized, for example when high or low temperatures are used or when there are problems with enzyme stability or inhibition (Richmond *et al.*, 1981; Portaccio *et al.*, 1998; Zhou & Chen, 2001; Mateo *et al.*, 2004).

The ascomycete *Trichoderma reesei* (*Hypocrea jecorina*) is used in industry to produce cellulolytic and hemicellulolytic enzymes. There is evidence that *T. reesei* produces at least two different β-galactosidases. The major β-galactosidase (Tr-β-gal) is a member of glycoside hydrolase (GH) family 35 and has a molecular weight of 109.3 kDa. The cloning, sequence analysis and enzymatic properties of this protein have previously been reported (Seiboth *et al.*, 2005). The protein shows activity towards several disaccharides, *e.g.* lactose, lactulose and galactobiose. It has a pH optimum at about 5.0 and a temperature optimum of 333 K for the hydrolysis (Gamauf *et al.*, 2007). To date, there are only three structures of β-galactosidases from GH family 35 available in the PDB. Two of these structures are from *Penicillium* sp. (Psp-β-gal): the native structure (PDB code 1tg7; Rojas *et al.*, 2004) and a structure in complex with galactose (PDB code 1xc6; Rojas *et al.*, 2004). The third structure, from *Bacteroides thetaiotamicron* (PDB code 3d3a, unpublished work), was reported by the New York Structural GenomiX Research Consortium. Psp-β-gal contains five domains and has been reported to have high transglycosylation activity (Neustroev *et al.*, 2000; Zinin *et al.*, 2002). Comparison of the Tr-β-gal and Psp-β-gal sequences showed 56% identity. Tr-β-gal is a potential enzyme for lactose hydrolysis in the dairy industry. An accurate three-dimensional structure of this enzyme would allow the design of mutations in order to improve its properties (such as pH optimum and stability). This paper presents the initial step towards the first three-dimensional structure of β-galactosidase from *T. reesei* (Tr-β-gal).

2. Materials and methods

2.1. Protein purification

The 1023-amino-acid full-length Tr-β-gal protein (GenBank accession No. AJ549427) was provided by Roal Oy (Rajamäki, Finland). It was overproduced in *T. reesei* similarly to the method described in Paloheimo *et al.* (2003). The Tr-β-gal protein was purified on an ÄKTA Purifier system (GE Healthcare). All steps were performed at room temperature (295 K), except for ammonium sulfate precipitation (280 K).

2.1.1. Step I: crude extract. *T. reesei* β-galactosidase was secreted into the culture medium, which was frozen and stored at 253 K after cultivation. 50 ml frozen culture medium was thawed and Complete Mini EDTA-free protease-inhibitor cocktail (Roche) was immediately added to the medium according to the manufacturer's instructions. Proteins were precipitated at 280 K overnight by ammonium sulfate [70%(*v*/*v*) of a saturated ammonium sulfate solution] and separated from the solution by centrifugation (7500 rev min^{-1} for 20 min). The centrifugated protein precipitate was then resuspended in buffer *A* {40 m*M* Tris–HCl pH 7.5 with ammonium sulfate [30%(*v*/*v*) of a saturated ammonium sulfate solution]}.

2.1.2. Step II: hydrophobic interaction chromatography (HIC). The protein solution was loaded onto a phenyl-Sepharose (GE Healthcare) column previously equilibrated with buffer *A*. After loading the protein solution, the column was washed with 100 ml buffer *A*. The protein was then eluted with a linear gradient of buffer *A* and buffer *B* (40 m*M* Tris–HCl pH 7.5).

2.1.3. Step III: ion-exchange chromatography. The β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (*o*PNG) as a substrate. A 20 µl fraction was added to 20 µl 10 m*M* *o*PNG. Hydrolysis of *o*NPG produced a yellow colour (released *o*NP) which indicated β-galactosidase activity.

The fractions with β-galactosidase activity were pooled and dialyzed against buffer *C* (20 m*M* sodium phosphate pH 7.6). The dialyzed protein solution was loaded onto a DEAE Sepharose Fast Flow (GE Healthcare) column previously equilibrated with buffer *C*. After loading the protein solution, the column was washed with 100 ml buffer *C*. The enzyme was then eluted using a linear gradient of buffer *C* and buffer *D* (20 m*M* sodium phosphate pH 7.6, 1 *M* NaCl).

Fractions with β-galactosidase activity were pooled and dialyzed against buffer *E* (10 m*M* Tris–HCl pH 7.5). The dialyzed solution was concentrated to a final protein concentration of at least 1 mg ml^{-1} and its purity was confirmed by SDS–PAGE electrophoresis. SDS–PAGE was performed in a 4–15%(*w*/*v*) gradient gel (Bio-Rad). Precision Plus Protein Standards (Bio-Rad) were used as molecular-mass standards and the proteins were detected by staining with Coomassie Brilliant Blue (Fig. 1).

2.2. Crystallization

Crystallization was performed using the hanging-drop vapour-diffusion technique at 295 K. A 2 µl drop of the protein solution (1 mg ml^{-1}) was mixed with an equal volume of the crystallization solution and equilibrated against 500 µl crystallization solution. Screening of the crystallization conditions was initiated with a Crystal Screening HT screening kit (Hampton Research).

Optimization of the crystallization conditions was performed by screening around the concentration of PEG and the pH value of the buffer solution. Crystals that grew in 8%(*w*/*v*) PEG 8000, 0.1 *M*

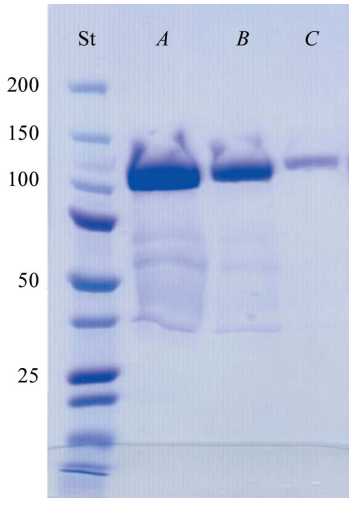

Figure 1
SDS–PAGE of purified *T. reesei* β-galactosidase. Lane *A*, protein sample (10 µg loaded); lane *B*, protein sample (5 µg loaded); lane *C*, protein sample (1 µg loaded). SDS–PAGE was performed in a 4–15%(*w*/*v*) gradient gel (Bio-Rad). Precision Plus Protein Standards (Bio-Rad) were used as molecular-mass standards (lane St; labelled in kDa) and the proteins were detected by staining with Coomassie Brilliant Blue.

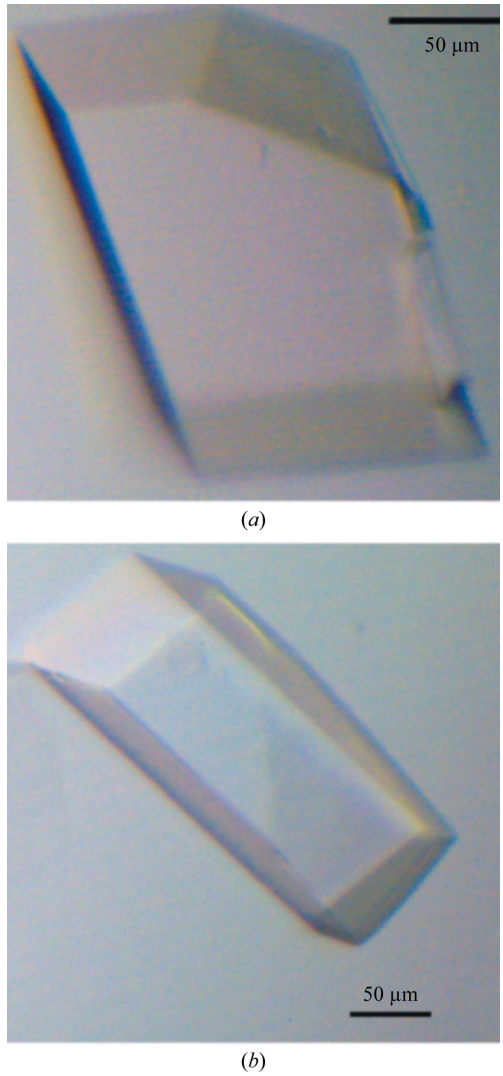

Figure 2
Tr-β-gal crystals used for data collection to 1.8 Å (*a*) and 1.2 Å (*b*) resolution. The scale bar represents 50 µm.

Table 1
Comparison of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	25–1.8 (1.9–1.8)	50–1.2 (1.3–1.2)
Wavelength (Å)	1.54 [rotating anode]	0.97926 [ID-29]
Temperature (K)	100	100
Space group	*P*1	*P*1
Unit-cell parameters (Å, °)	a = 67.4, b = 68.8, c = 81.6, α = 108.7, β = 97.3, γ = 114.8	a = 67.3, b = 69.1, c = 81.5, α = 109.1, β = 97.3, γ = 114.5
Total No. of reflections	272171 (39425)	719611 (142034)
No. of unique reflections	102728 (14850)	341073 (71839)
$R_{\rm merge}$† (%)	7.9 (35.5)	5.5 (44.3)
Average $I/\sigma(I)$	15.6 (4.36)	11.97 (3.28)
Completeness (%)	92.1 (89.0)	90.6 (89.4)
Mosaicity (°)	0.2	0.2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean value, respectively.

sodium cacodylate pH 6.0 crystallization solution were then used as seeds in the final optimization step. Homogeneous streak-seeding (using a dog hair) was performed after a 24 h pre-equilibration of the crystallization drops against 500 µl crystallization solution. These drops also contained 2 µl protein solution and 2 µl crystallization solution [8%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.0].

2.3. X-ray diffraction

The first X-ray data were collected to 1.8 Å resolution at the home laboratory on an FR591 rotating-anode source (Bruker Nonius) with a MAR Research Desktop Beamline goniometer, a MAR 345 imaging-plate detector (MAR Rsearch) and Osmic confocal optics. The cryosolution, 25%(*v*/*v*) glycerol in 0.1 *M* sodium cacodylate buffer pH 6.0 without PEG, was introduced by a quick soak (5 s). The crystal (Fig. 2*a*) was quickly vitrified in a gaseous nitrogen stream at 100 K with a Cryostream 700 (Oxford Cryosystems); the oscillation range was 1° and the distance between the detector and the crystal was 120 mm.

Subsequently, X-ray data were collected to 1.2 Å resolution from a second crystal (Fig. 2*b*) at a wavelength of 0.97926 Å on beamline ID-29 of ESRF (Grenoble, France). Owing to the high resolution, the data were collected in two passes. The distance between the detector and the crystal was 106.6 mm for the high-resolution data set and 425.8 mm for the low-resolution data set. The oscillation range was 0.5° and 1° for the high-resolution and low-resolution data sets, respectively. The diffraction data were processed using the *XDS* program and scaled with *XSCALE* (Kabsch, 1993).

3. Results and discussion

The purified protein solution contained both Tr-β-gal and several smaller polypeptides (Fig. 1) that could be impurities or degradation products from the cultivation. However, the intensities of the bands clearly showed that the solution mostly contained pure Tr-β-gal.

The initial screening produced protein crystals in only one condition: 0.1 *M* Tris–HCl pH 8.5 using 8%(*w*/*v*) PEG 8000 as a precipant (Crystal Screen HT screening kit). The crystals appeared to be disordered as evidenced by the lack of a visible diffraction pattern even at low resolution. Slightly larger crystals were obtained by changing the buffer to 0.1 *M* sodium cacodylate pH 6.0. These crystals diffracted to a maximum resolution of about 3.5 Å (data not shown). Therefore, homogenous streak-seeding was carried out. After 4–6 d, new rectangular crystals were obtained. The maximal dimensions of these crystals were about 0.1 × 0.1 × 0.3 mm. The crystals belonged to space group *P*1. Diffraction data sets were collected from one crystal on a rotating-anode X-ray home source and from a second single crystal using synchrotron radiation (Table 1). Calculation of the Matthews coefficient (Matthews, 1968) suggested the presence of one monomer per asymmetric unit ($V_{\rm M}$ = 3.16 Å^3 Da^{-1}) with a solvent content of 61.1%.

Structure determination is under way by molecular replacement using the Psp-β-gal structure (PDB code 1tg7) as a search model. The obtained 1.2 Å resolution data have the highest resolution available to date for a three-dimensional structure of β-galactosidases (previous structures had resolutions of 1.60–2.15 Å). We hope that these high-resolution data will provide new information concerning the structure and function of β-galactosidases and that they will form a starting point for rational protein engineering of the enzyme.

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